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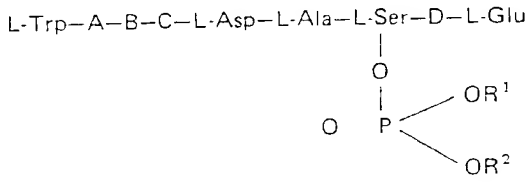
(54) **Phosphorylated Nonapeptides**

(57) Deep sleep-inducing agents are compounds of the formula



(I)

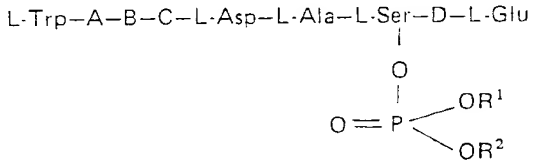
, wherein A represents *L*- or *D*-alanyl, B, C and D represent glycyl or *D*-alanyl and R^1 and R^2 represent hydrogen or alkyl,
and derivatives thereof in which one or more of the amide groups in N-alkylated and/or at least one carboxyl group of *L*-Asp and *L*-Gku is esterified, amidated or monoalkylamidated or dialkylamidated or reduced to a hydroxymethyl group in which the hydroxy group may again be acylated, phosphorylated or alkylated.

SPECIFICATION

Phosphorylated nonapeptides and a process for the manufacture thereof

The present invention is concerned with phosphorylated nonapeptides having valuable biological and pharmacodynamic properties and with a process for the manufacture thereof.

The phosphorylated nonapeptides provided by the present invention are compounds of the general formula



(I)

, wherein A represents *L*- or *D*-alanine, B, C and D represent Gly or *D*-alanine and R^1 and R^2 represent hydrogen or alkyl,
and derivatives of compounds of formula I in which one or more of the amide groups is N-alkylated and/or the terminal amino group is acylated or monoalkylated or dialkylated and/or at least one carboxyl group of *L*-Asp and *L*-Glu is esterified, amidated or monoalkylamidated or dialkylamidated or reduced to a hydroxymethyl group in which the hydroxy group may again be acylated, phosphorylated or alkylated.

A nonapeptide having the sequence L-Trp-L-Ala-Gly-Gly-*L*-Asp-*L*-Ala-*L*-Ser-Gly-*L*-Glu (molecular weight = 849), hereinafter referred to as DSIP (Delta-Sleep-Inducing Peptide) was isolated in the period 1971-1977 as the humoral sleep factor from the blood of rabbits, purified and characterised. Its structure was also finally elucidated (Pflügers Arch. *369*, 99-101, 1977). Biological tests carried out using synthetic DSIP gave identical results in comparison with natural DSIP isolated from rabbit blood when administered intra-cerebral-ventricularly in rabbits (Experientia *33*, 548, 1977). Intra-cerebral-ventricular doses of 6 nmol DSIP/kg induced a physiological effect in rabbits which, on an EEG, manifested itself as being equivalent to the phenomena found during natural orthodox deep sleep (Proc. Natl. Acad. Sci. USA *74*, 1282-1286, 1977). Synthetically prepared DSIP was also active following intravenous administration of 30 nmol/kg in cats and rats, whereby the EEG recordings and also the passive behavioural tests showed a significant improvement in deep and paradoxal sleep. The administration of synthetic DSIP by means of an arterial perfusion on isolated rat heads also gave EEG readings which were sleep equivalent. In animal trials, methods of application which may also be used in human medicine also resulted in the induction of a natural state of sleep.

It has, however, been shown in a series of experiments that the induction of sleep following the administration of DSIP results in an all or nothing effect when the dosage varies by only ± 20 nmol/kg body weight; that is to say, only the exact dosage o the correct quantity of DSIP achieves the desired effect. Furthermore, it has been found that intravenous administration requires a dosage of 6 nmol/kg which is substantially higher than that required for intra-cerebral-ventricular administration for the induction of sleep and, finally, if administration is carried out by the intravenous route, there is a delay before the induction of sleep.

These differences in activity following the two types of application are not surprising, since oligopeptides of the DSIP type are known from experience to undergo rapid enzymatic degradation on intravenous (subcutaneous or percutaneous) administration and, in addition, do not easily penetrate the blood brain barrier.

It is therefore an object of the present invention to provide compounds of the DSIP type which are more stable to enzymatic degradation, more easily penetrate the blood brain barrier and have improved biological and pharmacodynamic properties.

Comparative tests are set out hereinafter which show that DSIP which has been phosphorylated on the OH group of the serine and also the corresponding derivatives of DSIP analogues in which the second, third, fourth and/or eighth amino acid radical of the sequence is a *D*-alanine radical, on intravenous application to rats, not only give a reduction in the dosage needed, as compared with DSIP, but also give an accentuated activity with more rapid onset and longer (i.e. for more hours)duration. This protracted and accentuated activity constitutes an important therapeutic advantage.

Preferred phosphorylated nonapeptides provided by the present invention are compounds of formula I itself and, among such compounds, those in which A represent L-alanine and B, C and D represent glycine such as, for example:

L-Trp–L–Ala–Gly–Gly–L–Asp–L–Ala–L–Ser(O–PO(OH)$_2$)–Gly–L–Glu

and

L-Trp–L–Ala–Gly–Gly–L–Asp–L–Ala–L–Ser(O–PO(O-isopropyl)$_2$)–Gly–L–Glu

According to the process provided by the present invention, the phosphorylated nonapeptides aforesaid (i.e. the compounds of formula I and their derivatives as hereinbefore defined) are manufactured by phosphorylating a nonapeptide of the general formula

L-Trp–A–B–C–L-Asp–L-Ala –L-Ser–D–L-Glu (II)

,wherein A, B, C and D have the significance given earlier,
or a derivative of such a nonapeptide as hereinbefore defined, or splitting off the protecting group(s) from a compound of formula I, or a derivative thereof as hereinbefore defined, in which at least one of the functional groups is protected.

The individual reactions which lead to the phosphorylated nonapeptides of the present invention, their purification and the preparation of the starting materials may be carried out using conventional methods; that is to say, by using methods which are well known in peptide chemistry [see for example "Methoden der organischen Chemie" (Houben-Weyl) Vol. XV, parts 1 and 2, Georg Thieme Verlag, Stuttgart, 1974].

Thus, for example, the starting material used may be synthetically prepared DSIP or one of its analogues in positions 2, 3, 4 and/or 8; that is to say, a nonapeptide of formula II in which the functional groups, with the exception of the OH group of serine which is to be phosphorylated, are protected. The phosphorylation may be carried out by reaction with a monofunctional derivative of phosphoric acid such as dibenzyl phosphoryl chloride or diphenyl phosphoryl chloride. The protecting groups should be chosen so that they can be removed under mild conditions which do not result in the modification of the peptide sequence. Examples of protecting groups which may be used, since they may be removed by catalytic hydrogenolysis without difficulty, include N-benzyloxycarbonyl, N-benzyl, benzyl ester and ether groups. The protected nonapeptide may be phosphorylated in anhydrous pyridine using dibenzyl phosphoryl chloride. The phosphorylation product is washed, purified with an aqueous acid or base and subjected to hydrogenolysis in *tert*.butanol solution. The resulting phosphorylated nonapeptide may be purified by means of thin-layer chromatography or anion-exchange chromatography. The phosphorylation with diphenyl phosphoryl chloride is preferred in view of a selective splitting off of N-benzyl-oxycarbonyl and benzyl ester groups by means of palladium-catalysed hydrogenolysis. In order to remove one or both phenyl groups a platinum catalyst may be used [Acta. Chem. Scan. 13, 1407 and 1422 (1959)].

On the other hand, the starting material used may be an unprotected DSIP sequence which is phosphorylated with a monohalophosphoric acid [e.g. C1PO(OH)$_2$] or a monohalophosphoric acid ester [e.g. di*iso*propylfluorophosphate FPO[OCH(CH$_3$)$_2$]$_2$].

The following Examples illustrate the process provided by the present invention:

Example 1

Phosphorylation of N-benzyloxycarbonyl-DSIP-benzyl ester

A solution of N-benzyloxycarbonyl-DSIP-benzyl ester (5 nmol) dissolved in 10 ml of pyridine (dried over barium oxide) was cooled almost to freezing. Dibenzyl phosphoryl chloride (freshly prepared from dibenzyl) was then added, the mixture was well shaken and left to stand overnight at 4°C. 75 ml of cold ethyl acetate and 75 ml of cold water were then added to the mixture which was centrifuged. The supernatant was then successively washed with cold water, 1-*M* sulphuric acid, water, saturated sodium bicarbonate solution and water and dried over anhydrous sodium sulphate. The phosphate ester of the protected peptide was obtained in solid form after vacuum distillation of the solvent. The solid was then dissolved in a mixture of *tert*.butanol/water and hydrogenated using 10% palladium/carbon. The mixture was filtered, the catalyst and the filtrate were washed with water and the combined washings were evaporated to dryness under a vacuum. The thus-obtained DSIP, which was phosphorylated on the hydroxy group of the serine

[DSIP
|
O-PO(OH)$_2$,],

was purified using thin-layer chromatography or an anion-exchanger [Acta Chem. Scan. *15*, 163 (1961)].

Example 2

Phosphorylation of DSIP

5 mg of DSIP and 1 mg of *L*-tryptophan were mixed with 1 ml of phosphorus oxytrichloride at 5°C. The mixture was dissolved in 100 ml of concentrated formic acid and stirred for 8 hours at 0°C with the exclusion of moisture. The product was then lyophilised over sodium hydroxide under a high vacuum. After the addition of 2 ml of ice-water, the pH of the solution was adjusted to 8 at 0°C using 1-N sodium hydroxide. This pH was maintained constant for 2 hours. The solution was again lyophilised and the resulting lyophilisate was dissolved in 0.5 ml of water. 100µl portions of this solution were applied to a silica gel plate (layer thickness 2 mm, free from binding agent) and developed over a period of 8 hours at room temperature with acetone/water (7:3, v/v). The plate, apart from a strip round the edge, was covered with aluminium foil and sprayed with Florescamine solution (0.2% in acetone). Using UV light it was thus possible to fix the positions of the bands containing the desired material (Rf 0.47). These were scraped off and eluted with water. The supernatant resulting from the centrifugation of the eluate at 25,000 g was lyophilised. The thus-obtained material was dissolved in 0.5 ml of water and loaded on to a Sephadex (registered Trade Mark) G-15 column (145 ml) which was then eluted with water. Fractions of 1 ml were collected. The fractions which showed UV absorption at 280 nm were collected and lyophilised. The thus-purified and lyophilised material

[(DSIP
|
O—PO(OH)$_2$)]

was used for the rising test in rats [J. Biol. Chem. *202*, 67 (1953)].

Example 3

Phosphorylation of DSIP

10 mg of DSIP were dissolved in 0.5 ml of water and 0.1 ml of 1-*M* phosphate buffer pH 7.3. A 20 molar excess of di*iso*propylfluorophosphate was added to this solution which was then well stirred at room temperature for 30 minutes. A further 100µl of 1-*M* phosphate buffer (pH 7.3) were then added to this mixture which was then left to stand for a further 4 hours at room temperature. The mixture was then loaded on to a Sephadex G-15 column (145 ml) and eluted with water at room temperature. 100 fractions of 1.5 ml each were collected. The fractions which contained the phosphorylated DSIP

[(DSIP
|
O—PO(O-isopropyl)$_2$)]

(Nos. 35-45) were purified and lyophilised. The material was then used directly for the rising test in rats.

Example 4

20 mg of DSIP (23.5µmol) in 3 ml of phosphorus oxytrichloride was dissolved in 500µl of formic acid at 0°C. The mixture was then stirred at 0°C for 200 hours with the complete exclusion of moisture. Excess phosphorus oxytrichloride and formic acid were then removed under a high vacuum. In order to remove the chlorine atoms of the phosphate ester radical, the material was dissolved in 5 ml of ice-water and stirred at pH 8 for 2 hours at 0°-4°C. The material was finally lyophilised.

The lyophilised crude product was then dissolved in 1.2 ml of water and applied to 6 plates for preparative thin-layer chromatography (silica gel), in each case 2 cm from the edge of the plate. Development was carried out for 8 hours at room temperature using acetone/water (7:3, v/v). The plates were covered with aluminium foil apart from a strip round the edge 3 cm wide and sprayed with Florescamine solution (0.2% in acetone). Illumination of the plates with UV light at 350 nm showed specs round the edge. The bands having an Rf value of 0.6 were indicated, removed and eluted with water. The eluates were centrifuged at 20,000 g for 10 minutes and the supernatant was lyophilised.

The lyophilised peptide was dissolved in 2 ml of water, loaded on to a Sephadex G-15 column (220 ml) and eluted with water.

The evaluation of the fractions was carried out using UV light at 280 nm and showed a peak with a flat shoulder. The fractions belonging to the peak were divided into 3 pools and lyophilised separately. The [illegible] Sephadex G-15 and again lyophilised.

The material of pool was identified as practically 100% DSIP phosphorylated on the OH group of the serine. It had a Rf value on silica gel plates in a water/acetone (7:3, v/v) system of 0.42. The non-phosphorylated starting material showed under similar conditions a Rf value of 0.87. Yield 25%.

Determination of biological activity in the rising test

In a randomised double blind study the vigilant state in 8 test rats and 8 control animals was simultaneously quantified. In the rising test, each rising of a test animal was counted as a point. The experiment was carried out 90 minutes after the intravenous injection of the substance (Test, T) in 0.2 ml of sodium chloride solution or of 0.2 ml of sodium chloride solution alone (Control C), for 3 hours during the alert period of the rats before midnight. The counting was carried out by three unbiased people, again under randomised double blind conditions. The frequency of the rising of the control animals was set at 100% and the reduction of the number of risings of the test animals is calculated. The results are summarised in the following Table.

No.	Dose (nmol/kg)	Compound	C (%)	T (%)
1	90	DSIP	100	54
2	10	DSIP * O—PO(OH)$_2$	100	45
3	30	DSIP* O—PO(O-isopropyl)$_2$	100	50

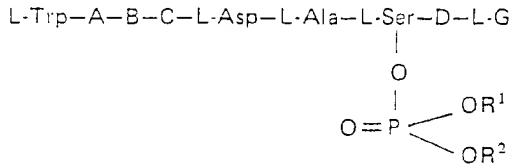
Test versus control $p < 0.001$

*Phosphorylation on serine radical

The phosphorylated nonapeptides provided by the present invention may be used in the form of pharmaceutical preparations, having a direct or delayed release of the active ingredient, which contain them in admixture with suitable organic or inorganic inert carrier materials which are suitable for enteral or parenteral administration such as for example water, gelatin, gum arabic, lactose, starches, magnesium stearate, talc, vegetable oils, polyalkyleneglycols or petroleum jelly. The pharmaceutical preparations may be made up in a solid form (e.g. tablets, dragées or capsules) or in a liquid form (e.g. as solutions, suspensions or emulsions). If desired, the pharmaceutical preparations can be sterilised and/or can contain adjuvants such as preserving agents, stabilising agents, wetting or emulsifying agents, agents for improving taste, salts for varying the osmotic pressure or buffers. The pharmaceutical preparations may be prepared in a conventional manner.

CLAIMS

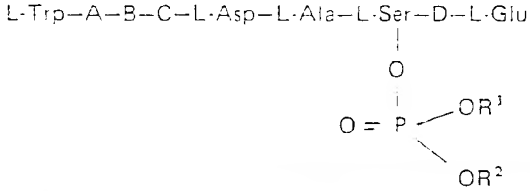
1. Compounds of the general formula



(I)

, wherein A represents *L*- or *D*-alanine, B, C and D represent Gly or *D*-alanine and R^1 and R^2 represent hydrogen or alkyl,
and derivatives of compounds of formula I in which one or more of the amide groups is N-alkylated and/or the terminal amino group is acylated or monoalkylated or dialkylated and/or at least one carboxyl group of *L*-Asp and *L*-Glu is esterified, amidated or monoalkylamidated or dialkylamidated or reduced to a hydroxymethyl group in which the hydroxy group may again be acylated, phosphorylated or alkylated.

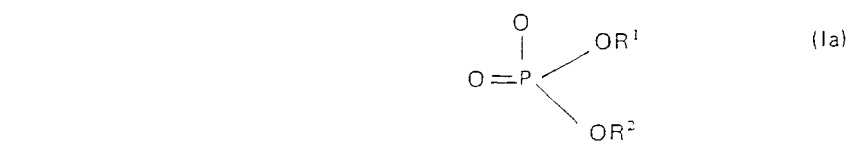
2. Compounds of the general formula



(I)

wherein A represents *L*- or *D*-alanine, B, C and D represent Gly or *D*-alanine and R^1 and R^2 represent hydrogen or alkyl.

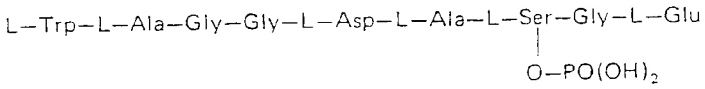
3. Compounds of the general formula



(Ia)

wherein R^1 and R^2 represent hydrogen or alkyl.

4.



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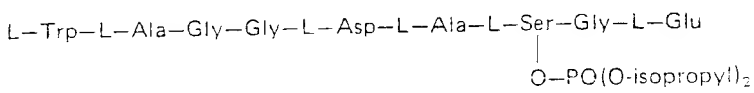
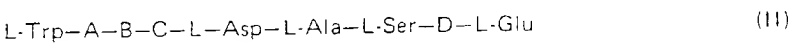


6. A process for the manufacture of the phosphorylated nonapeptides claimed in claim 1, which process comprises phosphorylating a nonapeptide of the general formula



(II)

, wherein A, B, C and D have the significance given in claim 1, or a derivative thereof as defined in claim 1, or removing protecting groups on at least one of the functional groups of a compound of formula I or of a derivative thereof as hereinbefore defined.

7. A process according to claim 6, wherein the phosphorylation is carried out by reaction with phosphoric acid, a phosphoric acid ester, a phosphoric acid halide, a phosphoric acid halide ester or a pyrophosphate.

8. A process according to claim 7, wherein the phosphorylation is carried out using chlorophosphoric acid or di*iso*propylfluorophosphate.

9. A process for the manufacture of the phosphorylated nonapeptides set forth in claim 1, substantially as hereinbefore described with reference to the foregoing Examples.

10. Phosphorylated nonapeptides as set forth in claim 1, when manufactured by the process claimed in any one of claims 6 to 9 inclusive or by an obvious chemical equivalent thereof.

11. A pharmaceutical preparation containing a phosphorylated nonapeptide as defined in claim 1.

12. A process for the preparation of a pharmaceutical preparation, which process comprises mixing a nonapeptide as set forth in claim 1 with a thereapeutically acceptable inert solid or liquid carrier and, if desired, bringing the mixture obtained into a galenic form.

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